Ligations-Troubleshooting

I always do a PCR purification after digestion -- even if I am doing a gel extraction -- before I do my ligation. After ligation, I would also do another digest with a single cut enzyme that will cut your vector (not your insert), and run on a gel to verify the size. Run it next to vector alone (cut with the same single cut enzyme) and you should see the 500bp difference to make sure your insert is ligated properly. Then you can proceed with the transformation.

LIGASE-TEST

In order to test your ligase, try addingsome to DNA ladder (in ligase buffer, of course). Treat at RT for 15-20 minutes, then run on a gel. If you don't have a change in the ladder (everything sfited up), your ligase is a (the?) problem.

➔ Usual ratio Vektor: Insert =1:3

Increase your vector to insert ratio. 1:4, 1:5...

But by sure restrictase was right inactivated by temperature!!!!

OR:

Do not use kit for purification but do it old way, ethanol precipitation. Ligation ratio should by 1:3 vector insert, since ligation is bimoleculaar reaction ,but 1:5 increase chance for success

• incubate at 16°C for difficult ligations, increase ligation time

http://openwetware.org/wiki/DNA_Ligation

10µL Ligation Mix

Larger ligation mixes are also commonly used

- 1.0 µL 10X T4 ligase buffer
- 6:1 molar ratio of insert to vector (~10ng vector)
- Add (8.5 - vector and insert volume)µl ddH_2O
- 0.5 µL T4 Ligase

Achtung, Ligase-Puffer ist sehr instabil, ATP können bei längerem RT kaputt gehen!!!

1. Remember that the buffer contains ATP so repeated freeze, thaw cycles can degrade the ATP thereby decreasing the efficiency of ligation.

1. Denature the ligase at 65°C for 10min
2. Dialyze for 20 minutes if electroporating

Ligation efficiency was **marginally decreased** by

1. Doing a 1 hr ligation at room temperature
2. Using 100 ng vector
3. Using insert:vector molar ratios of 5:1 and 1:1

Ligation efficiency was **noticably decreased** (x100) by

1. Sticky end ligation with a larger insert (5.2 kb vector + 2.6 kb insert)
2. Blunt end ligation

Ligation efficiency was **severely decreased** (x10000) by

1. Using DNA fragments that have been exposed to UV during the gel extraction procedure (*can avoid by blind excision, or by using a black-light or 365nm UV transilluminator instead of the usual 312nm type*)
2. Using the NEB Quick Ligation Kit (*heat inactivation of PEG in the buffer ruins transformation, without heat inactivation the ligation probably would've been fine*)

PEG promotes ligation but inhibits transformation

For additional troublshooting, check out the NEB FAQ page for T4 ligation: [1]
http://www.neb.com/nebecomm/products/faqproductM0202.asp#339

3. **A1:** * Ligation failed because there was no ATP or Mg2+. Use the supplied buffer or add ATP to a compatible buffer. The ATP in buffers older than one year may have degraded enough to cause problems. When supplementing with ATP, be sure to use ribo ATP as deoxyribo ATP will not work.
   * Ligation failed due to high salt or EDTA in the reaction. Clean up the DNA.
   * CIP, BAP or SAP not completely inactivated from dephosphorylation step. Follow the recommended procedure to remove the phosphatase.
   * Ligation produced only linear DNA because the DNA concentration was too high. Keep the total DNA concentration between 1-10 µg/ml.
4. *Too much ligation mixture was added to the cells. Add between 1-5 µl to 50 µl competent cells
5. *The ligation mix contained PEG and was incubated overnight. Extended ligation with PEG causes a drop off in transformation efficiency. This could be due to the gradual production of large linear pieces of DNA that can inhibit transformation. The buffer for the Quick Ligation Kit (NEB# M2200) contains PEG.
6. The ligation mix was not purified prior to electroporation. The buffer must be removed or a spark will be generated by the salt. Dialyse the sample or use a spin column to purify. The PEG in the Quick Ligation Kit Buffer (NEB# M2200) prevents sparking but it also prevents electroporation. PEG must be removed using a spin column.
   Q9: Can T4 DNA Ligase be heat inactivated?

   A9: Yes, heat at 65 °C for 20 minutes. Do not heat inactivate if there is PEG in thereaction buffer (Quick Ligation Kit buffer (NEB# M2200) because transformation will be inhibited.